Vaccination Against *Staphylococcus aureus* Pneumonia

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**Background.** *Staphylococcus aureus* causes serious infections in both hospital and community settings. Attempts have been made to prevent human infection through vaccination against bacterial cell-surface antigens; thus far all have failed. Here we show that superantigens and cytolysins, when used in vaccine cocktails, provide protection from *S. aureus* USA100–USA400 intrapulmonary challenge.

**Methods.** Rabbits were actively vaccinated (wild-type toxins or toxoids) or passively immunized (hyperimmune serum) against combinations of superantigens (toxic shock syndrome toxin 1, enterotoxins B and C, and enterotoxin-like X) and cytolysins (α-, β-, and γ-toxins) and challenged intrapulmonarily with multiple strains of *S. aureus*, both methicillin-sensitive and methicillin-resistant.

**Results.** Active vaccination against a cocktail containing bacterial cell-surface antigens enhanced disease severity as tested by infective endocarditis. Active vaccination against secreted superantigens and cytolysins resulted in protection of 86 of 88 rabbits when challenged intrapulmonarily with 9 different *S. aureus* strains, compared to only 1 of 88 nonvaccinated animals. Passive immunization studies demonstrated that production of neutralizing antibodies was an important mechanism of protection.

**Conclusions.** The data suggest that vaccination against bacterial cell-surface antigens increases disease severity, but vaccination against secreted virulence factors provides protection against *S. aureus*. These results advance our understanding of *S. aureus* pathogenesis and have important implications in disease prevention.

**Keywords.** *Staphylococcus aureus*; vaccination; pneumonia; endocarditis; superantigens; cytolysins.

*Staphylococcus aureus* causes many infections, from benign skin and soft tissue infections to life-threatening pneumonia, infective endocarditis, and sepsis [1, 2]. The ability of *S. aureus* to cause an array of infections results from the large number of virulence factors produced, both cell-surface and secreted [1, 2]. Among the secreted virulence factors, superantigens and cytolysins are critical participants in infections, and these exotoxins are associated with significant morbidity and mortality [2, 3]. Treatment of life-threatening *S. aureus* infections is costly, requiring hospitalization, with lengthy antibiotic treatment and in many cases, surgery [1, 4].

There is no effective vaccine against *S. aureus*. A number of vaccine trials have been initiated, but the trials have failed [5]. The last trial ended prematurely, as infections and deaths in vaccinated people were higher than nonvaccinated control subjects [5]. Although the precise reason for the vaccine failures is unknown, there is evidence to suggest that the failures resulted in part from use of bacterial cell-surface virulence factors as vaccine candidates. Organisms such as *S. aureus* and *Enterococcus faecalis* aggregate in their hosts, with consequent increased virulence [6–8]. Vaccination to generate immunoglobulin G (IgG) against aggregation substance, an aggregation-inducing surface protein of *E. faecalis*, results in enhanced infective endocarditis severity and lethality in vaccinated animals compared to nonvaccinated animals [9]. Passive administration of IgG Fab fragments against aggregation substance reduces disease severity, demonstrating that
IgG-enhanced aggregation caused the increased virulence in actively vaccinated animals [9]. We hypothesized the same may happen with *S. aureus*.

This study was undertaken to evaluate active vaccination and passive immunization against key secreted virulence factors to protect rabbits from fatal pneumonia. We also assessed the role of vaccination against cell-surface virulence factors in enhancing infective endocarditis severity in rabbits.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth**

Toxic shock syndrome toxin 1 (TSST-1) [10] and TSST-1 toxoid G31S/S32P (lacks binding to MHC II) [11], staphylococcal enterotoxin B (SEB) and SEB toxoid Q210A (lacks T-cell receptor binding) [12], staphylococcal enterotoxin C (SEC) and SEC toxoid N23A (lacks T-cell receptor binding) [13], wild-type SEI-X [14], and α-toxin toxoid H35A were purified from *S. aureus* strain RN4220 expressing individual genes from a plasmid. Strain RN4220 does not produce endogenous superantigens. RN4220 was also used as the source of wild-type β-toxin. *Staphylococcus aureus* strain MNJA and MNPE were sources of wild-type α-toxin. *Escherichia coli* clones with pET-30(a) were the sources of the γ-toxin B chain. Vaccination against *S. aureus* surface proteins was performed with cell-wall preparations from strain ATCC12598.

*Staphylococcus aureus* strains used in pneumonia challenge studies are listed in Table 1. The strains belong to pulsed-field gel electrophoresis clonal groups USA100–USA400. All strains have the genes for α, β, and γ-toxins, but USA200 strains MNPA, MN8, and CDC587 have a stop codon within the α-toxin structural gene, reducing α-toxin production by 50-fold. All strains have the capacity to produce β-toxin, but in nearly all non-USA200 strains, the β-toxin gene is disrupted by bacteriophages. These bacteriophages excise and are lost variably among non-USA200 strains. The superantigens in Table 1 do not include all superantigens genes carried by the strains; those listed include only superantigens relevant to protection studies.

For intrapulmonary administration, organisms were grown overnight in 25 mL of Todd Hewitt broth (Difco Laboratories) at 37°C with shaking at 200 revolutions per minute. The organisms were washed once with phosphate-buffered saline (PBS) followed by centrifugation at 20 800 g for 5 minutes, and then resuspended in Todd Hewitt broth at 2.5–4.0 × 10^8 cells/0.4 mL for high-dose injection.

For production of a surface protein vaccine, ATCC12598 was cultured to stationary phase in RPMI 1640 medium, which is limited in iron; iron limitation causes upregulation of genes required for bacterial iron transport. Thus, iron-regulated surface determinants become expressed in greater amounts. Subsequently, the cells were washed once in PBS and resuspended to an absorbance at 600 nm wavelength of 1.0 in 50 mM Tris buffer at pH 7.3, containing 20 mM magnesium chloride. The cells were then treated simultaneously with lysostaphin (200 µg/mL) and lysozyme (25 mg/mL) for 30 minutes to disrupt the cell walls. Insoluble cell debris was removed by centrifugation (10 000 g, 30 minutes). The resultant clarified supernate was adjusted to 200 µg/mL of protein, and 1 mL per injection was used for vaccination of 5 rabbits.

<table>
<thead>
<tr>
<th>Strain* (Resistance)</th>
<th>Clonal Group by PFGE</th>
<th>Cytolysinsb</th>
<th>Superantigens</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA209 (MSSA)c</td>
<td>USA100</td>
<td>α, β, γ-toxins</td>
<td>SEI-X</td>
<td>Dr A. R. Horswill, University of Iowa</td>
</tr>
<tr>
<td>MNPE (MSSA)</td>
<td>USA200</td>
<td>α, β, γ-toxins</td>
<td>TSST-1, SEC</td>
<td>Postinfluenza pneumonia and TSS [15]</td>
</tr>
<tr>
<td>MNPA (CA-MRSA)</td>
<td>USA200</td>
<td>α, β, γ-toxins</td>
<td>TSST-1</td>
<td>Menstrual TSS</td>
</tr>
<tr>
<td>MN8 (MSSA)</td>
<td>USA200</td>
<td>α, β, γ-toxins</td>
<td>TSST-1, SEC</td>
<td>Menstrual TSS [16]</td>
</tr>
<tr>
<td>CDCB87 (MSSA)</td>
<td>USA200</td>
<td>α, β, γ-toxins</td>
<td>TSST-1, SEC</td>
<td>Menstrual TSS [17]</td>
</tr>
<tr>
<td>LAC CA-MRSA</td>
<td>USA300</td>
<td>α, β, γ-toxins</td>
<td>SEI-X</td>
<td>F. R. Deleo, NIH</td>
</tr>
<tr>
<td>MNLe MSSA</td>
<td>USA300</td>
<td>α, β, γ-toxins</td>
<td>SEI-X</td>
<td>Necrotizing pneumonia</td>
</tr>
<tr>
<td>MW2 CA-MRSA</td>
<td>USA400</td>
<td>α, β, γ-toxins</td>
<td>SEC, SEI-X</td>
<td>Necrotizing pneumonia</td>
</tr>
<tr>
<td>C99-529</td>
<td>USA400</td>
<td>α, β, γ-toxins</td>
<td>SEB, SEI-X</td>
<td>Necrotizing pneumonia</td>
</tr>
</tbody>
</table>

Abbreviations: CA, community associated; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; NIH, National Institutes of Health; PFGE, pulsed-field gel electrophoresis; SEB, staphylococcal enterotoxin B; SEC, staphylococcal enterotoxin C; TSS, toxic shock syndrome; TSST, toxic shock syndrome toxin.

* Resistance refers to the presence or absence of methicillin-resistance as determined by the presence or absence of SCCmec DNA.

* USA200 strains produce wild-type amounts of β-toxin (approximately 500 µg/mL). Other clonal groups variably produce β-toxin dependent on excision of the β-toxin gene-inactivating bacteriophage; all of these strains produce some β-toxin.

* Strain IA209 was chosen as a representative USA100 strain based on testing 12 independent strains belonging to this clonal group. All 12 strains were positive for α, β, and γ-toxins and the superantigen SEI-X; 1 strain produced SEC.

* These strains have their α-toxin gene disrupted by a stop codon. They thus produce approximately 1/50th the amount of strains with the wild-type gene. Wild-type strains typically produce α-toxin at 5–100 µg/mL.

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Secreted Virulence Factor Purification

All reagents used in preparation of superantigens were pyrogen-free. For production of TSST-1 and toxoids, SEB and toxoids, SEC and toxoids, SE-X, and wild-type α-toxin, S. aureus RN4220 was grown overnight in a dialyzable beef-heart medium [18]. The exoproducts were precipitated from culture fluids with absolute ethanol (80% final concentration), resolubilized in water, and purified by thin-layer isoelectric focusing [18]. The resultant proteins were homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10 µg stained with Coomassie blue R250) and assays for hemolysins, lipase, nucleases, and proteases [19]. Wild-type α-toxin was prepared comparably except that the initial toxin precipitation step utilized 80% ammonium sulfate [20]. Toxin was resolubilized in water and excess ammonium sulfate removed by dialysis for 3 days. The resultant protein was homogeneous by SDS-PAGE (10 µg stained with Coomassie blue R250) and assays for hemolysins, lipase, nucleases, and proteases. All purified proteins reacted as expected in Western immunoblots with hyperimmune antisera raised against the purified toxin. Unless otherwise noted, all proteins were quantified using the Bio-Rad assay (Bio-Rad Corporation) with SEB as the standard.

The γ-toxin nontoxic B chain was produced in E. coli, where expression was induced in Luria broth (kanamycin 30 µg/mL) using 0.3 mM isopropyl β-D-1-thiogalactopyranoside. Bacterial pellets were lysed with lysozyme (100 µg/mL) and sonication. The lysate supernate was sterile-filtered and incubated with Ni-NTA resin (Invitrogen) to bind the B chain. The protein was eluted from the resin using an imidazole gradient.

The α-toxin H35A protein was prepared from supernates of overnight cultures of RN4220. Supernates were collected by centrifugation at 4°C at 3200g, 15 minutes, filtered through a 0.22 µm Sterilip-GP filter unit (Millipore), and concentrated using the Microcon Centrifugal Filter Device YM-30 (Millipore). The concentration of the prepared proteins was determined by using a BCA Protein Assay Kit (Pierce).

Rabbits

Rabbit use adhered to approved protocols established by the Universities of Iowa and Minnesota Institutional Animal Care and Use Committees. New Zealand White rabbits, male and female, weighing 2–3 kg were used. Rabbits were purchased from Bakkom Rabbitry.

Vaccinations

Rabbits were vaccinated against biologically inactivated (toxoid) proteins, β-toxin, and the B chain of γ-toxin by emulsifying 25 µg in Freund incomplete adjuvant [11]. Vaccinations were administered in multiple subcutaneous sites in the nape of the necks. Native superantigens and cytotoxins were used for vaccinations at sublethal doses of approximately 10 µg/mL, following the same vaccination protocol. Vaccinations for all experiments were performed every other week for a total of 3 injections. One week after the final vaccination, blood was drawn from the marginal ear veins, serum samples were collected, and antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) [11]. In brief, 96-well plates (NUNC Maxisorp) were coated with 1.0 µg/well of purified native homologous superantigen or cytotoxin and then washed (SE-X was coated at a concentration of 10 µg/well). Rabbit serum samples were serially diluted 2-fold; plates were incubated for ≥1.5 hours at room temperature, and then washed. Horseradish peroxidase–conjugated antirabbit IgG antibodies (Sigma-Aldrich) were added to the wells. The plates were again incubated for ≥1.5 hours, and the wells were washed. IgG levels were determined by addition of o-phenylenediamine and hydrogen peroxide. Reactions were stopped by the addition of 50 µL of 12.5% sulfuric acid, and absorbance was determined at 490 nm wavelength.

Rabbit Pulmonary Illness and Infective Endocarditis Model

Rabbits were administered bacteria (2.0–4.0 × 10^7 colony-forming units [CFUs] in 0.4 mL volumes) via intratracheal inoculation [21]. Rabbits were anesthetized with ketamine (25 mg/kg) and xylazine (25 mg/kg) (Phoenix Pharmaceuticals). Their necks were shaved, and small incisions were made to expose the tracheae. Incisions were made into the tracheae followed by insertions of 1-mm diameter polyethylene catheters (Becton, Dickinson, and Co), threading them into the left bronchi. Microbes were administered through the catheters, the catheters removed, and incision sites closed. Rabbits were monitored for 7 days for development of pneumonia, toxic shock syndrome (TSS) symptoms (fever, difficulty breathing, diarrhea, reddening of conjunctivae, and evidence of hypotension), and lethality. Rabbits were prematurely euthanized when they simultaneously failed to remain upright and could not exhibit flight responses expected of wild rabbits when approached by humans. Animals were euthanized with 1 mL/kg of Beuthanasia-D (Schering-Plough). Surviving rabbits were killed at the end of 7 days.

We compared the ability of intravenous immunoglobulin (IVIG; 12 000 µg twice daily; ZLB Bioplasma AG) and the same volume of PBS to protect rabbits from SEB intrapulmonary lethality. Animals (5 per group) were anesthetized and 200 µg SEB was administered intratracheally. The animals were closed and IVIG or PBS was injected intravenously at time 0, 24, or 48 hours relative to SEB. Animals were monitored for survival for 4 days. The typical progression of illness when treated with PBS was to show temperatures of ≥41°C on day 1 (baseline temperatures 38.7°C), temperatures of approximately 37°C on day 2, suggestive of animals progressing into shock, and death by day 3.

For infective endocarditis, rabbits were anesthetized and their left carotids exposed [22]. Catheters were placed in the carotids until they abutted the aortic valves; they remained in
place for 2 hours. Subsequently, the catheters were removed, the incision sites closed, and the animals injected intravenously with *S. aureus* MW2 (1 × 10⁷ CFU). Animals were monitored for up to 4 days for survival and development of vegetations.

**Statistics**

Log-rank and Fisher exact tests were used to compare differences in animal survival. Comparison of antibody titers between groups was accomplished using the Student *t* test or among groups with analysis of variance.

**RESULTS**

To establish whether increased lethality in rabbits would be obtained through active vaccination against bacterial cell-surface components of *S. aureus* as we had seen previously with *E. faecalis* infective endocarditis [9], we prepared an extract of bacterial cell-surface components that was enriched for iron surface determinants, vaccinated rabbits, and then challenged the vaccinated and control rabbits in a comparable infective endocarditis model. Rabbits were vaccinated against *S. aureus* cell-surface virulence factors, including protein A, iron surface determinants, and clumping factor A or remained nonvaccinated. Animals (5 per group) were vaccinated every other week for a total of 3 injections, shown to have ELISA antibody titers of >10 000 against the pool of antigens, and then challenged with viable organisms. All 5 vaccinated animals succumbed to intravenous challenge with community-associated methicillin-resistant *S. aureus* (CA-MRSA) MW2 in <6 hours (Figure 1).

In contrast, all 5 nonimmune animals survived for the entire 4-day test period, and all developed vegetations. These data are in agreement with our prior findings, which suggested that bacterial aggregation by IgG antibodies enhances virulence in this model [9].

We subsequently adopted a novel strategy focusing on vaccination against secreted virulence factors; antibodies against these factors should not enhance aggregation. A prior study [23] showed that vaccination of mice against staphylococcal α-toxin protected mice from lethal necrotizing pneumonia. However, mice are resistant to the lethal effects of superantigens, which are produced in high levels by all pathogenic *S. aureus* strains, and mice are moderately resistant also to the lethal effects of α-toxin [24]. Rabbits are the most useful animal model for the study of *S. aureus* diseases, as these animals have susceptibilities to superantigens and cytolysins similar to humans [24]. Thus, rabbits were vaccinated with monovalent or polyvalent antigens composed of superantigens and cytolysins, and challenged with a 100x lethal intrapulmonary dose of multiple *S. aureus* strains from 4 different clonal groups [25] (USA100, USA200, USA300, and USA400), both methicillin-sensitive *S. aureus* (MSSA) and MRSA. We chose to evaluate vaccination against pneumonia, as it is a serious *S. aureus* illness affecting as many as 70 000 persons in the United States annually [26]. Collectively, our studies demonstrate successful protection of 86 of 88 vaccinated rabbits, compared to 1 of 88 nonvaccinated animals (*P* < .0001 by Fisher exact test; Table 2).

We tested the ability of a cocktail of 7 secreted virulence factors to provide protection from lethal intrapulmonary challenge with a representative USA100 strain (IA209). (USA100 strains [12 tested] produce the superantigen SEJ-X and the cytolysins α, β, and γ-toxins; 1 strain tested also produces SEC.) USA100 organisms commonly colonize nasal passages and are a cause of hospital-associated infections [27]. Five of 6 vaccinated rabbits survived lethal challenge at 7 days, compared to 0 of 6 nonvaccinated animals (Table 2). Importantly, surviving animals did not develop signs of pulmonary disease, except fever. In contrast, animals that succumbed showed fevers, diarrhea, weight loss, and hypotension, progressing to shock and death. Examination of lungs after experimentation indicated that surviving animals had minor pulmonary damage, grossly visible only as small granulomas and without viable bacteria (Figure 2). In contrast, animals that succumbed had severe necrotizing (hemorrhagic) pneumonia.

The rabbits started with average antibody titers of ≤33 for all toxins tested. After vaccination, rabbits had high antibody titers.
against all toxins tested, whereas nonvaccinated animals had low antibody titers (Figure 3).

To evaluate further whether the generation of antibody is the primary mechanism of protection in vaccinated rabbits, we passively vaccinated naive rabbits and challenged the animals intrapulmonarily with strain MNPE [15]; this bacterial strain produces TSST-1 and SEC superantigens and wild-type amounts of $\alpha$-toxin, $\beta$-toxin, and $\gamma$-toxin [20]. The hyperimmune serum used for passive immunization was taken from the vaccinated animals used in the above experiment (postvaccination sera; Figure 3). Pooled vaccinated animal serum (3 mL) was injected intravenously into naive rabbits 15 minutes prior to pneumonia challenge. Control rabbits were injected with 3 mL pooled serum from nonvaccinated rabbits.

All rabbits that received serum from vaccinated animals survived to the end of the 7-day experiment (Figure 4); these animals remained healthy, and their lungs appeared normal. In contrast, 5 of 6 rabbits that received control serum from nonvaccinated animals died before the end of day 7 (Figure 4). All of the rabbits showed signs of TSS.

<table>
<thead>
<tr>
<th>Clonal Group</th>
<th>Strain</th>
<th>Vaccination Against</th>
<th>Vaccinated Survival</th>
<th>Nonvaccinated Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA100</td>
<td>IA209</td>
<td>TSST-1, SEB, SEC, SE/E/X, $\alpha$-toxin, $\beta$-toxin, $\gamma$-toxin</td>
<td>5/6$^a$</td>
<td>0/6</td>
</tr>
<tr>
<td>USA200</td>
<td>MNPE</td>
<td>TSST-1, SEC, $\alpha$-toxin</td>
<td>11/11$^a$</td>
<td>0/11</td>
</tr>
<tr>
<td></td>
<td>CDC587</td>
<td>TSST-1, SEC, $\alpha$-toxin</td>
<td>8/8</td>
<td>0/8</td>
</tr>
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<td></td>
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<td>TSST-1</td>
<td>8/8</td>
<td>1/8</td>
</tr>
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<td></td>
<td>MN8</td>
<td>TSST-1, SEC, $\alpha$-toxin</td>
<td>8/8$^a$</td>
<td>0/8</td>
</tr>
<tr>
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<td>LAC</td>
<td>TSST-1, $\alpha$-toxin</td>
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<td>0/8</td>
</tr>
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<td></td>
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<td>8/8</td>
<td>0/8</td>
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<tr>
<td></td>
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<td>0/8</td>
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<td>USA400</td>
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<td>SEB, $\alpha$-toxin</td>
<td>8/8$^a$</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td>MW2</td>
<td>SEC, $\alpha$-toxin</td>
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<td>0/8</td>
</tr>
<tr>
<td></td>
<td>MW2</td>
<td>SEC</td>
<td>6/7</td>
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<tr>
<td>Total No. of rabbits, survived/total</td>
<td>86/88</td>
<td>1/88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collective $P$ value $= 1 \times 10^{-46}$ by Fisher exact test.

Abbreviations: SEB, staphylococcal enterotoxin B; SEC, staphylococcal enterotoxin C; TSST, toxic shock syndrome toxin.

* Animals in these challenge groups were evaluated for colony-forming units (CFU) in the lungs compared to their respective nonvaccinated controls. No CFUs were demonstrated in the lungs of vaccinated animals after 7 days compared to $>10^9$ CFU in nonvaccinated animals at the time of death.

Figure 2. Example lungs from animals that were nonvaccinated vs vaccinated against secreted virulence factors. The animals were challenged intrapulmonarily with $2-4 \times 10^8$ colony-forming units of *Staphylococcus aureus* IA209. Animals were examined for lung status upon premature euthanasia in the case of the nonvaccinated rabbit or upon termination of the experiment in the case of the vaccinated animal. Two, small sterile granulomas are visible on the lung from the vaccinated rabbit, as was typically seen in all such animals.
We had insufficient immune rabbit serum from vaccinated animals to assess the ability of immunoglobulins to passively protect animals that had been administered bacteria and then had delayed treatment. Our vaccine studies in Table 2 showed that 22 of 23 rabbits actively vaccinated with a single superantigen were protected from lethal challenge, compared to 1 of 23 control nonvaccinated animals ($P < .0001$). These data indicate that immunity to superantigens is critical for survival in the rabbit model. Given these findings, we tested the ability of commercially available human IVIG to protect rabbits from lethal intrapulmonary challenge with purified SEB (Figure 5). Simultaneous administration or delayed administration of IVIG by 24 hours still resulted in 100% survival of rabbits compared to 0% protected when given PBS ($P = .008$ at both time points). Delaying IVIG administration even for 48 hours resulted in increased survival compared to PBS treatment (4 of 5 survived vs 0 of 5; $P = .05$). The typical course of disease observed in PBS treated animals is high fever by day 1, body temperature reduced significantly below normal by day 2 (suggestive of progression to shock), and death by day 3. For animals treated with simultaneous or delayed IVIG and surviving, their disease progression was the same as PBS-treated animals until IVIG was administered. Within 24 hours, their temperatures returned to normal. By day 4, surviving animals appeared healthy and were eating.

For USA200 strains, a total of 35 rabbits were vaccinated with either a trivalent vaccine (27 rabbits; TSST-1, SEC, and α-toxin) or a monovalent vaccine (8 animals; TSST-1) (Table 2). The animals were challenged with 4 different USA200 strains, 3 from menstrual TSS patients and 1 from a fatal case of TSS and...
pneumonia [15–17, 19]. Only 1 of 35 of the vaccinated animals succumbed to lethal intrapulmonary challenge with these *S. aureus* strains. All remained healthy over the 7-day test period. In contrast, 34 of 35 nonvaccinated animals succumbed in <7 days with comparable challenge.

For USA300 strains, a total of 24 rabbits were vaccinated with either a bivalent vaccine (16 rabbits; TSST-1, α-toxin) or a monovalent vaccine containing TSST-1 (8 animals) (Table 2). The animals were challenged with 2 different USA300 strains, 1 strain was CA-MRSA [28] and the other MSSA (fatal necrotizing pneumonia, and extreme pyrexia [29]). None of the 24 vaccinated animals succumbed to lethal intrapulmonary challenge, remaining healthy over the 7-day test period. In contrast, 24 of 24 nonvaccinated control animals succumbed in response to comparable intrapulmonary challenge.

For USA400 strains, 23 rabbits were vaccinated with a bivalent vaccine (8 rabbits with SEB and α-toxin and 8 rabbits with SEC and α-toxin) or a monovalent vaccine (7 animals; SEC) (Table 2). The animals were challenged with 2 different USA400 strains, 1 producing SEB and 1 producing SEC, and both strains were CA-MRSA from fatal infections [30]. All vaccinated rabbits survived lethal intrapulmonary challenge. All surviving animals remained healthy over the 7-day test period. In contrast, 23 of 23 nonvaccinated animals died when given comparable challenge.

**DISCUSSION**

We evaluated the ability of vaccination against native toxins and toxoids to protect a sensitive animal model from lethal pneumonia. It is clear that human vaccination with use of these molecules would require nontoxic vaccine toxoids. We have developed such toxoids in the course of these studies. Importantly, our studies collectively show that vaccination against secreted virulence factors, whether native toxin or toxoids, provides protection against lethal intrapulmonary challenge with multiple *S. aureus* strains and clonal groups, both MSSA and MRSA. In contrast, immunization against a cocktail of antigens containing bacterial cell-surface virulence factors enhanced infective endocarditis severity. The data suggest that with multidimensional pathogens, such as invasive and toxigenic *S. aureus*, vaccination against secreted virulence factors may be required to prevent diseases.

We protected rabbits from lethal pneumonia through use of selected superantigens and cytolysins as antigens. We employed SEI-X in only 1 set of studies, those using USA100 *S. aureus*. It is interesting that USA300 strains do not contain the typical high-level superantigens [31], instead producing SEI-X as their dominant superantigen [14]. Vaccination did not require the presence of SEI-X for complete protection against USA300 isolates. This may have occurred for 2 reasons: (1) antibodies to SEI-X appear to cross-react with TSST-1, which was used in vaccination cocktails, and (2) USA300 strains produce high levels of the cytolysin α-toxin, which likely contributes significantly to disease production [23].

Importantly, there are 2 major differences between our vaccine studies as tested in rabbits compared to prior vaccine studies, as tested in mice and then nonhuman primates. We chose superantigens and cytolysins as the basis for our vaccine. Superantigens and cytolysins are important in human diseases due to *S. aureus* [1, 2]. We anticipated that neutralization of these exotoxins would prevent serious diseases and lethality but would not affect colonization. However, our studies indicate, that when tested, rabbits were developing sterilizing immunity by 7 days postchallenge, indicating the organisms were not able to colonize the lungs long term. This suggests vaccination against these secreted virulence factors may prevent colonization. Superantigens and cytolysins are not required for microbiologic growth. Thus, it is unlikely that the formation of neutralizing antibodies results in direct killing of the organisms, but instead, significantly reduces the ability of *S. aureus* to evade the immune system, allowing the organisms to be more easily phagocytosed and killed. In prior vaccine studies including human trials, the major targets of vaccination were bacterial cell-surface factors that aid in colonization or are required for iron acquisition [5]. Importantly, use of these factors for vaccination and possible aggregation by IgG enhances acute microbial virulence, suggesting that immunization by this method may be ineffective.

The second major difference between our studies and prior studies is our use of rabbits as the prehuman trial model system. Rabbits, like humans, are highly susceptible to both superantigens and cytolysins [24]. Prior studies developed their human vaccines using mice and nonhuman primates, which are resistant to superantigens and moderately resistant to cytolysins [24, 32]. Our studies demonstrate that *S. aureus* strains (nearly 8000 tested) from serious human illnesses produce high levels of superantigens, and all produce at least 1 high-level cytolysin, most often α-toxin. Thus, the prior studies disregarded a major *S. aureus* virulence factor in human infections.

Collectively, our work demonstrates that vaccination of rabbits against exoproteins provides protection (86 of 88) against intrapulmonary challenge with exceptionally high doses of both MSSA and MRSA from USA100–USA400 lineages. Only 1 nonvaccinated animal survived equivalent challenge. These data suggest that vaccination with superantigens and cytolysins, against all strains of *S. aureus*, will provide protection since the major secreted virulence factors of *S. aureus* strains are similar to those produced by USA100–USA400 lineages. Furthermore, our studies demonstrate that development of toxin-neutralizing antibodies is an important mechanism of vaccine protection, although we have not assessed if cellular immunity, as contributed by CD4 T cells activating macrophages or CD8 T cells, functions in immunity.
Notes

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Potential conflicts of interest. P. M. S. and M. L. P. are coinventors on a patent application related to these studies filed by the University of Minnesota. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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